High mobility group box 1 promotes angiogenesis from bone marrow-derived endothelial progenitor cells after myocardial infarction

High mobility group box 1 は骨髄由来血管内皮前駆細胞を誘導し心筋梗塞後の血管新生を促進する

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論　文　内　容　要　旨

【背景】High mobility group box 1 (HMGB1)は DNA 結合タンパクであり、核内で遺伝子の安定化を担っている。しかし、HMGB1 は組織障害時に細胞外に放出され、receptor for advanced glycation end products (RAGE)や Toll-like receptor を介して炎症細胞に作用し、炎症性生理活性物質の産生や傷害組織を修復するサイトカイン様の働きを示すと報告されている。HMGB1 心筋特異的過剰発現マウス(HMGB1-TG)に心筋梗塞(MI)を作成したところ、心筋傷害が軽減され心機能が改善した。本研究では HMGB1-TG マウスを用いて、HMGB1 と MI 後の骨髄由来細胞による血管新生の関連について検討した。【方法・結果】Green fluorescent protein (GFP)マウスの大腿骨から骨髄細胞を採取し、9 Gy の放射線を照射した野生型(WT)マウスと HMGB1-TG マウスに骨髄細胞を尾静脈から投与した。骨髄移植 2 週間後に開胸し左前下行枝を結紮した MI 群と、開胸のみで冠動脈を結紮しない sham 群を作成した。MI 作成 4 日後の末梢血中の HMGB1 濃度を ELISA にて測定したところ、HMGB1-TG-MI マウスでは WT-MI マウスに比べ HMGB1 濃度は高値であった。MI 作成後の心筋の HMGB1 濃度は梗塞巣と境界域では低値であり、梗塞巣の HMGB1 が血中に流出したものと考えられた。MI 作成 1 週後の末梢血中の血管内皮前駆細胞(EPCs)を flow cytometry にて検討した。MI 後に EPCs は WT、HMGB1-TG マウスともに増加したが、HMGB1-TG マウスでより増加していた。MI 作成 4 週後に心臓を摘出し組織学的検討を行った。HMGB1-TG マウスでは WT マウスより梗塞巣は縮小していた。免疫染色では抗 platlet endothelial cell molecule (PECAM)1 抗体および抗α-smooth muscle actin 抗体を用い検討したところ、陽性細胞数は HMGB1-TG マウスで WT マウスより多く、capillary と arteriole density が増加していた。蛍光免疫検査では GFP と PECAM1 の double positive cell である骨髄由来の新生血管内皮細胞の心臓細胞に対する比は HMGB1-TG マウスで WT マウスに比し高値であった。

MI 作成 1 週間後の心筋内の VEGF の濃度を ELISA で測定した。HMGB1-TG マウスでは梗塞周囲、遠隔域でともに WT マウスに比較し VEGF が高値であった。さらに、HMGB1-TG マウスで VEGF2 レセプターの発現が増加していた。

【結語】HMGB1 が骨髄細胞から EPCs を動員し、梗塞巣に遊走させ梗塞後の心臓内で血管内皮細胞として生着させることで血管新生を促進していることが考えられた。

Abstract

Aims: High mobility group box 1 (HMGB1) is a DNA-binding protein secreted into extracellular space from necrotic cells and acts as a cytokine. We examined the role of HMGB1 in angiogenesis from bone marrow-derived cells in the heart, using transgenic mice with cardiac-specific overexpression of HMGB1 (HMGB1-TG).

Methods and Results: HMGB1-TG mice and wild-type littermate (WT) mice were lethally irradiated and injected with bone marrow cells from green fluorescent protein mice through the tail vein. After bone marrow transplantation, the left anterior descending artery was ligated to create myocardial infarction (MI). Flow cytometry analysis revealed that circulating endothelial progenitor cells (EPCs) mobilized from bone marrow increased after MI in HMGB-TG mice compared with WT mice. The size of MI was smaller in HMGB1-TG mice than in WT mice. Immunofluorescence staining demonstrated that the numbers of engrafted vascular endothelial cells derived from bone marrow in the border zone of MI were increased in HMGB1-TG mice rather than in WT mice. Moreover, the levels of the cardiac vascular endothelial growth factor after MI were higher in HMGB1-TG mice than in WT mice.

Conclusions: The present study demonstrated that HMGB1 promoted angiogenesis and

reduced MI size by enhancing mobilization and differentiation of bone marrow cells to EPC, migration to the border zone of MI, and engraftment as vascular endothelial cells of new capillaries or arterioles in the infarcted heart.

Key words: HMGB1, myocardial infarction, angiogenesis, endothelial progenitor cell, VEGF

Introduction

Myocardial infarction (MI), which is caused by occlusion of the coronary artery, is the leading cause of heart failure and cardiac death [1]. Angiogenesis plays an important role in the repair of injured tissues, including MI [2]. Endothelial cells originating from bone marrow have been reported to participate in angiogenesis, and the mechanisms of angiogenesis after MI have been extensively studied [3-6].

High mobility group box 1 (HMGB1) is a DNA-binding protein located in the nucleus of almost all types of cells. It is a 215 amino acid protein and has important functions in stabilizing nucleosomes and facilitating DNA transcription, replication and repair [7–10]. HMGB1 is important for maintaining correct transcriptional control through specific transcription factors. A previous study has reported that HMGB1 knockout mice show hypoglycemia and die within a short time after birth as a result of impairing their glucocorticoid receptor; therefore, HMGB1 is essential for their survival [11]. HMGB1 is also known to be secreted into extracellular space from necrotic, but not apoptotic, cells and activated inflammatory cells, and to act as a cytokine [12]. Extracellular HMGB1 mediates inflammation and enhances regeneration of damaged

tissues [8, 13–15]. We have recently demonstrated that HMGB1 attenuates cardiac damage and restores cardiac function by enhancing angiogenesis after MI [16]. Moreover, HMGB1 induces myocardial regeneration by enhancing the proliferation of cardiac C-kit+ progenitor cells after MI [17].

In this study, we examined the role of HMGB1 in angiogenesis from bone marrow-derived cells in the heart after MI, using transgenic mice with cardiac-specific overexpression of HMGB1 (HMGB1-TG).

Methods

Animals and ethics statement

HMGB1-TG mice [16] and wild-type littermate (WT) mice (C57BL/6, male), 10 to 12 weeks old, were used for experiments. The investigations conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication, 8th Edition, 2011). Our research protocol was approved by the Fukushima Medical University Animal Research Committee, and all animal experiments were conducted in accordance with the guidelines of the Fukushima Medical University Animal Research Committee. All efforts were made to minimize suffering animals.

Bone marrow transplantation (BMT)

Green fluorescent protein (GFP) mice (C57BL/6, male), 6 to 12 weeks old (CLEA Japan, Inc., Tokyo, Japan), were lightly anesthetized by titrating isoflurane (0.5-1.5%) and sacrificed by cervical dislocation. Whole bone marrow cells from GFP mice were harvested by flushing their femurs with PBS as previously reported [18]. Briefly, the

cells were washed 2 times with PBS and diluted in 5.0 x 10^6 cell/200 μl with saline. The recipient mice (WT mice and HMGB1-TG mice, 8 to 10 weeks old) were lethally irradiated with a total dose of 9 Gy and injected with bone marrow cells through the tail vein.

Ligation of left anterior descending coronary artery

Induction of MI was performed as described previously [19]. Briefly, 2 weeks after bone marrow transplantation (BMT), WT and HMGB1-TG mice were anesthetized by intraperitoneal injection with a tribromoethanol (0.25 mg/g of body weight). The mice were intubated with a 20-gauge polyethylene catheter and were ventilated with a rodent ventilator (Shinano Manufacturing, Tokyo, Japan). An incision was made along the left sternal border, and the fourth rib was cut proximal to the sternum. The left anterior descending coronary artery was identified, and an 8–0 proline suture was passed around the artery and subsequently tied off. Successful ligation of the coronary artery was verified visually by the discoloration of the left ventricular myocardium. In sham-operated animals, the same procedure was performed except for the coronary

artery ligation. Finally, the heart was repositioned in the chest, and the chest wall was closed. The animals remained in a supervised setting until becoming fully conscious.

Flow cytometry analysis

Peripheral blood samples were collected from the mice at 2 weeks after BMT and 1 week after MI operation. Circulating cells were identified using a nucleated fraction determined by forward and side scatters.

EPCs are a subpopulation of the non-neutrophil cell lineage. First, we gated these cell lineages using anti-granulocyte differentiation antigen-1 (GR1). EPCs exhibit nonspecific hematopoietic stem cell and/or progenitor cell markers (CD34, CD117, or AC133), as well as specific endothelial markers (CD144, or CD146). CD34 is a marker for hematopoietic progenitor cells that give rise to all blood cells [20], and CD144 (VE-cadherin) is expressed on endothelial cells [21]. For that reason, we used CD34 and CD144 surface markers to identify EPCs in the flow cytometric analyses [22]. The cells, which were labeled with pacific blue-conjugated anti-CD34 (eBioscience, Inc., San Diego, CA, USA), R-phycoerythrin-conjugated anti-CD144 (eBioscience, Inc.), and

allophycocyanin-conjugated anti-GR1 (eBioscience, Inc.), were examined by a flow cytometer (BD FACSCanto II, BD Biosciences, San Jose, CA, USA) and analyzed with Flow Jo software version 7.6.2 (Tomy Digital Biology, Co., Ltd., Tokyo, Japan) to identify EPCs in GFP-positive donor bone marrow-derived cells.

Enzyme-linked immunosorbent assay (ELISA)

Plasma concentrations of HMGB1 at 4 days after MI operation in WT and HMGB1-TG mice were measured by ELISA kit (Shino-Test Corporation, Tokyo, Japan) according to the manufacturer's instructions, as previously reported [16].

Histological examinations

To assess morphological changes, the mice were weighed and sacrificed by cervical dislocation 4 weeks after MI operation. Their hearts were excised and weighed, and each heart was embedded in paraffin or optimal cutting temperature compounds. The tissues were sliced serially at the papillary muscle level of the mitral valve. These slices in paraffin were stained with hematoxylin-eosin stain and Masson–trichrome stain. In

each left ventricular transverse section, the infarct length was calculated by measuring the endo- and epi-cardial surface length delimiting the infarcted region. The percentage of the infarct size was calculated as the infarct length divided by the total left ventricular circumference, as previously reported [16, 23]. We took the digital photomicrographs and determined the left ventricular area using Image J software (version 1.38, National Institutes of Health). Frozen tissue sections 7 μm in thickness were cut on a cryostat and air-dried, and immunohistological staining was performed. We used anti-platelet endothelial cell adhesion molecule (PECAM1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to identify endothelial cells. The sections were stained with horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain Mouse MAX PO, Nichirei Biosciences, Inc., Tokyo, Japan) and diaminobenzidine etrahydrochloride, and counterstained with haematoxylin. We also used anti-α smooth muscle actin (αSMA) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to identify αSMA-positive vessels. The sections were stained with Histofine Simple Stain Mouse MAX PO and diaminobenzidine etrahydrochloride, and with haematoxylin. To assess the numbers of PECAM1 and αSMA-positive cells, we took digital

photomicrographs from the border zone (1–2 mm from the edge of the infarction zone) at 4 weeks after MI. Ten random microscopic fields in the border zone were examined, the numbers of PECAM1-positive cells/high power field (HPF, x 400) and αSMA-positive cells/HPF were counted, and the data from 10 fields were averaged [16].

We used anti-GFP (Abcam, plc., Cambridge, UK) antibody as a primary antibody, and goat anti-chicken IgY antibodies conjugated with Alexa Fluor488 (Abcam, plc.) as a secondary antibody. Furthermore, we used anti-PECAM1 (Santa Cruz Biotechnology, Inc.) and goat anti-rabbit IgG conjugated with Alexa Fluor594 (Santa Cruz Biotechnology, Inc.). Samples were observed by fluorescence microscopy with nuclear counter staining with 4’ 6-diamidino-2-phenylindole (DAPI). We took the digital photomicrographs of whole sections at 200× magnification. We calculated the ratio of GFP and PECAM1 double-positive cells, which were bone marrow-derived cells differentiated into vascular endothelial cells within the myocardial tissue, to total nuclei numbers. We also assessed the ratio of GFP/PECAM1 double-positive cells per PECAM1 positive cells.

Proteins in the left ventricle after myocardial infarction

The mice were sacrificed by cervical dislocation and their hearts were rapidly excised for the analyses at 1 week after MI operation. Proteins were extracted from the snap-flozen left ventricle in the remote and border zone of the MI with ice-cold lysis buffer, as previously reported [19, 23, 24]. The protein concentration of myocardial samples was carefully determined by the protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal concentrations of the protein solution (10 μg/μl) were analyzed using HMGB-1 ELISA kit (Shino-Test Corporation), vascular endothelial growth factor (VEGF) ELISA kit (Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan), and angiopoietin-1 ELISA kit (Cloud-Clone Corp., Houston, TX, USA) according to the manufacturer's instructions.

Extraction of total RNA and polymerase chain reaction (PCR)

The mRNA expression of VEGF receptor 2 in mouse hearts was determined by reverse transcription-PCR. Total RNA was extracted from mouse hearts using RNeasy Mini Kit

(Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The concentrations of all RNA samples were determined spectrophotometrically. The cDNA was produced from total RNA using ReverTra Ace qPCR RT Master Mix (ToYoBo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions, and PCR was performed using GeneAmpr PCR System 9700 (Applied Biosystems, Santa Clara, CA.). The expression level of VEGF receptor 2 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold increase over the levels in the sham-operated WT mice. Primers were designed on the basis of GenBank sequences (VEGF receptor 2, NM0106122 and GAPDH, NM001001303).

Statistical analysis

All results are expressed as mean ± standard error (S.E.). Statistical significance was evaluated with the unpaired *t*-test for comparisons between 2 groups. EPC and proteins in the left ventricle were analyzed by one-way analysis of variance (ANOVA), followed by multiple comparisons with Bonferroni test. A *p* value less than 0.05 was considered

statistically significant. These analyses were performed using a statistical software package (SPSS ver. 21.0, IBM, Armonk, NY, USA).

Results

Circulating EPCs counts

Flow cytometry showed that peripheral leukocytes steadily consisted of more than 90% of the GFP-positive cells at 2 weeks after BMT in both WT mice and HMGB1-TG mice. GFP-positive cells were identified as donor bone marrow cell-derived EPCs if they were positive for both CD34 and CD144 in the GR1-negative fraction. No significant difference was observed in the ratio of EPCs to GR1-negative cells between WT mice and HMGB1-TG mice before MI operation (Figs. 1A, 1B, and 1E). The ratio of EPCs to GR1-negative cells significantly increased in WT and HMGB1-TG mice at 1 week after MI operation ($p < 0.05$ and $p < 0.01$, respectively, Figs. 1A-1E). However, this increase was significantly higher in HMGB1-TG mice than in WT mice ($p < 0.01$), as shown in Fig. 1E.

Plasma and cardiac concentrations of HMGB1

We measured plasma concentrations of HMGB1 4 days after MI operation (Fig. 2A). The plasma HMGB1 level was not different between WT and HMGB1-TG

sham-operated mice, however, it was higher in HMGB1-TG MI mice than in WT MI mice ($p < 0.05$).

In contrast, the cardiac protein level of HMGB1 was much lower in the border zone of MI of the HMGB1-TG mice than in the remote zone of the HMGB1-TG mice and the sham-operated HMGB-TG mice (14.3 ± 2.9, 31.3 ± 5.0 and 30.1± 1.8 ng/μg protein, $p < 0.05$ and $p < 0.01$, respectively) as shown in Fig. 2B. These data suggest that HMGB1 was released into circulation from necrotic cardiomyocytes after coronary artery ligation.

Gravimetric data and left ventricular remodeling after MI

The body weight at 4 weeks after MI operation was not different between the WT and HMGB1 TG mice (23.8 ± 0.66 g vs. 24.6 ± 0.79 g). The ratio of heart weight to body weight at 4 weeks after MI operation was lower in HMGB1-TG mice than in WT mice (7.56 ± 0.30 mg/g vs. 8.65 ± 0.38 mg/g, $p < 0.05$).

We calculated the percentage of infarct size 4 weeks after coronary ligation (Fig. 3). The infarct size was significantly smaller in the HMGB1-TG than in the WT mice (*p*

< 0.05).

We also examined the numbers of PECAM1 and αSMA-positive cells from the border zone at 4 weeks after MI to measure capillary and arteriole densities (Fig. 3C and 3E). Numbers of PECAM1-positive cells were greater in the HMGB1-TG mice than in the WT mice (54.4 ± 2.8 vs. 38.3 ± 3.2, $p < 0.01$) as shown 3D. Numbers of αSMA-positive cells were greater in the HMGB1-TG mice than in the WT mice (10.2 ± 0.8 vs. 7.4 ± 1.1, $p < 0.05$) as shown 3F.

Myocardial immunofluorescence staining

Bone marrow-derived cells were confirmed by staining with GFP-specific antibody (Figs. 4A and 4F), and endothelial cells were confirmed by staining with PECAM1-specific antibody (Figs. 4B and 4G). Nuclei were confirmed by staining with DAPI (Figs. 4C and 4H). GFP and PECAM1 double-positive cells were bone marrow-derived cells engrafted within myocardial tissue as vascular endothelial cells of new capillaries or arterioles (Figs. 4D and 4I). The ratio of GFP and PECAM1-double positive cells to all cardiac cells was significantly higher in the HMGB1-TG mice than

in the WT mice ($p < 0.01$), as shown in Fig. 4K. The ratio of GFP/PECAM1 double positive cells per PECAM1 positive cells was higher in the HMGB1-TG mice than in the WT mice (42.2% vs. 31.6%, $p < 0.05$) as shown in Fig. 4L.

Cardiac levels of VEGF and angiopoietin after MI

As shown in Fig. 5A, levels of myocardial VEGF in the MI-operated mice were higher than those in the sham-operated mice in both WT and HMGB1-TG mice ($p < 0.05$ and $p < 0.01$, respectively). Furthermore, cardiac levels of VEGF in the HMGB1-TG MI mice were significantly higher than in the WT MI mice both in the border and remote zones of MI ($p < 0.05$, respectively), as shown in Fig. 5A. In contrast, cardiac angiopoietin levels were higher in the border zone of MI than in the remote zone in both HMGB1-TG and WT mice, and there was no difference between HMGB1-TG and WT mice (Fig. 5B). These data suggest that HMGB1 enhanced angiogenesis through increasing the myocardial VEGF level.

Expression of VEGF receptor 2 gene after MI

We examined the mRNA expression of VEGF receptor 2 (Fig. 6). The expression levels of VEGF receptor 2 were higher in the MI mice (border and remote zones) than in the sham-operated mice. Furthermore, the mRNA expression levels of VEGF receptor 2 in border and remote zones were higher in the HMGB1-TG mice than those in the WT mice ($p < 0.05$ and $p < 0.01$, respectively).

Discussion

The present study showed direct *in vivo* evidence that HMGB1 supported bone marrow-derived cells engrafted within myocardial tissue as vascular endothelial cells of new capillaries or arterioles, enhanced angiogenesis, and subsequently reduced MI size after coronary artery ligation.

HMGB1 was identified as a chromatin-binding protein with important structural functions for stabilizing chromatin organization [8–10]. Also, extracellular HMGB1 secreted from activated inflammatory cells responded to tissue damage by acting as a cytokine [12]. Increase of the extracellular HMGB1 level is reported in the subacute phase after cell injury [25]. Extracellular HMGB1 binds to receptor for advanced glycation end products (RAGE), toll-like receptors (TLR)-2, TLR-4 and TLR-9 [26]. These signals lead to activation of nuclear factor-κB and mitogen-activated protein kinase, [27–29] and enhance angiogenesis and regeneration of injury tissues so as to activate cell migration and cytokine secretion [26, 28, 29]. Chavakis et al. have reported that HMGB1 stimulates EPC migration and promotes the homing to ischemic tissues [30]. Rossini et al. have reported that HMGB1 significantly enhances growth factors

including VEGF in cultured cardiac fibroblasts [31].

RAGE is a multi-ligand receptor for advanced glycation end products constituting a major group of ligands. Mitola et al. suggested that HMGB1 and RAGE on endothelial cells might contribute to angiogenic activity [32]. RAGE mediates endothelial cell activation and smooth muscle cell migration. Schlueter et al. indicated that HMGB1 activated macrophage, following release of VEGF via nuclear factor-κB signaling [33]. Moreover, resident endothelial cells proliferate and migrate in response to stress or inflammation [34]. These responses might be enhanced in the heart with higher levels of HMGB1 and VEGF. VEGF reportedly stimulates the release of stromal cell-derived factor-1α, whose expression in infarcted hearts results in mobilization and homing of bone marrow stem cells [35]. These reports generally support our finding of an increased number of EPCs in peripheral circulation, and the high number of vascular endothelial cells derived from bone marrow cells in the border zone of MI in the HMGB1-TG mouse heart. HMGB1 released from the infarcted myocardial area might mobilize bone marrow-derived cells to damaged cardiac tissue and promote proliferation and differentiation into EPC and vascular endothelial cells.

It has also been reported that HMGB1 has beneficial effects in low doses but deleterious effects in high doses [36]. Since HMGB1 levels in our HMGB1-TG mice might be relatively low level, HMGB1 showed beneficial angiogenic effects to enhance recovery from cardiac injury. For these reasons, HMGB1 may have an important role for improving angiogenesis after MI. Consequently, the size of MI and the ratio of heart weight to body weight were reduced in HMGB1-TG mice compared with WT mice. HMGB1 enhances angiogenesis by the intermediation of bone marrow-derived cells, and prevents cardiac remodeling after MI.

Conclusions

The present study demonstrated in *in vivo* mouse hearts that HMGB1 promoted angiogenesis through enhancing the mobilization and differentiation of bone marrow cells to EPC, migration of EPC to the ischemic border zone, and engraftment as the vascular endothelial cells in the infarcted myocardium.

Acknowledgments

We thank Drs. Satoshi Suzuki, Takeshi Shimizu, Makiko Miyata, Tetsuro Shishido, Dr. Kazuhiko Ikeda, Shu-ichi Saitoh, Isao Kubota and Yasuchika Takeishi for their out standing support. We thank also Ms. Emiko Kaneda and Minae Takasaki for their excellent technical assistance. This study was supported in part by a grant-in-aid for Scientific Research (No. 24591100) from Japan Society for the Promotion of Science and grants-in-aid from the Japanese Ministry of Health, Labor, and Welfare, Tokyo, Japan.

This paper was published in Journal of Atherosclerosis and Thrombosis (epub ahead).

Conflict of Interest

None declared.

References

1. Gheorghiade M, Sopko G, de Luca L, Velazquez EJ, Parker JD, et al. (2006) Navigating the crossroads of coronary artery disease and heart failure. Circulation 114: 1202–1213.

2. Carmeliet P, Jain RK. (2000) Angiogenesis in cancer and other diseases. Nature 407: 249–257.

3. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, et al. (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 18: 3964–3972.

4. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, et al. (2001) Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 104: 1046–1052.

5. Fuchs S, Baffour R, Zhou YF, Shou M, Pierre A, et al. (2001) Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional

function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 37: 1726–1732.

6. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, et al. (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 107: 1395–1402.

7. Stros M. (2010) HMGB proteins: interactions with DNA and chromatin. Biochim Biophys Acta 1799: 101–113.

8. Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. (2002) HMGB1 as a DNA-binding cytokine. J Leukoc Biol 72: 1084–1091.

9. Goodwin GH, Sanders C, Johns EW. (1973) A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem 38: 14–17.

10. Bianchi ME, Beltrame M. (2000) Upwardly mobile proteins. The role of HMG proteins in chromatin structure, gene expression and neoplasia. EMBO Rep 1: 109–119.

11. Calogero S, Grassi F, Aguzzi A, Voigtländer T, Ferrier P, et al. (1999) The lack of

chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. Nat Genet 22: 276–280.

12. Yang H, Wang H, Czura CJ, Tracey KJ. (2005) The cytokine activity of HMGB1. J Leukoc Biol 78: 1–8.

13. Scaffidi P, Misteli T, Bianchi ME. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418: 191–195.

14. Yanai H, Ban T, Taniguchi T. (2012) High-mobility group box family of proteins: ligand and sensor for innate immunity. Trends Immunol 33: 633–640.

15. Popovic PJ, DeMarco R, Lotze MT, Winikoff SE, Bartlett DL, et al. (2006) High mobility group B1 protein suppresses the human plasmacytoid dendritic cell response to TLR9 agonists. J Immunol 177: 8701–8707.

16. Kitahara T, Takeishi Y, Harada M, Niizeki T, Suzuki S, et al. (2008) High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice. Cardiovasc Res 80: 40–46.

17. Limana F, Germani A, Zacheo A, Kajstura J, Di Carlo A, et al. (2005) Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction

via enhanced cardiac C-kit+ cell proliferation and differentiation. Circ Res 97: 73–83.

18. Yajima N, Takahashi M, Morimoto H, Shiba Y, Takahashi Y, et al. (2008) Critical role of bone marrow apoptosis-associated speck-like protein, an inflammasome adaptor molecule, in neointimal formation after vascular injury in mice. Circulation 117: 3079–3087.

19. Takeishi Y, Huang Q, Abe J, Glassman M, Che W, et al. (1999) Src and multiple MAP kinase activation in cardiac hypertrophy and congestive heart failure under chronic pressure-overload: comparison with acute mechanical stretch. J Mol Cell Cardiol 33: 1637–1648.

20. Morrison SJ, Uchida N, Weissman IL. (1995) The biology of hematopoietic stem cells. Rev Cell Dev Biol 11: 35-71.

21. Breier G, Breviario F, Caveda L, Berthier R, Schnürch H, et al. (1996) Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. Blood 87: 630-641.

22. Ozdogu H, Sozer O, Boga C, Kozanoglu L, Maytalman E, Guzey M. (2007) Flow

cytometric evaluation of circulating endothelial cells: a new protocol for identifying endothelial cells at several stages of differentiation. Am J Hematol 82: 706-711.

23. Takahashi H, Takeishi Y, Miyamoto T, Shishido T, Arimoto T, et al. (2004) Protein kinase C and extracellular signal regulated kinase are involved in cardiac hypertrophy of rats with progressive renal injury. Eur J Clin Invest 34: 85–93.

24. Takeishi Y, Abe J, Lee JD, Kawakatsu H, Walsh RA, et al. (2004) Differential regulation of p90 ribosomal S6 kinase and big mitogen-activated protein kinase-1 by ischemia/reperfusion and oxidative stress in perfused guinea pig hearts. Circ Res 85: 1164–1172.

25. Hirata Y, Kurobe H, Higashida M, Fukuda D, Shimabukuro M, et al. (2009) HMGB1 plays a critical role in vascular inflammation and lesion formation via toll-like receptor 9. Atherosclerosis 231: 227–233.

26. Lotze MT, Tracey KJ. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5: 331–342.

27. Autschbach F, Pleger ST, Lukic IK, Bea F, Hardt SE, et al. (2008) High-mobility group box-1 in ischemia-reperfusion injury of the heart. Circulation 117: 3216–3226.

28. Biscetti F, Ghirlanda G, Flex A. (2011) Therapeutic potential of high mobility group box-1 in ischemic injury and tissue regeneration. Curr Vasc Pharmacol. 9: 677-681.

29. Yamada S, Maruyama I. (2007) HMGB1, a novel inflammatory cytokine. Clin Chim Acta 375: 36–42.

30. Chavakis E, Hain A, Vinci M, Carmona G, Bianchi ME, et al. (2007) High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells. Circ Res 100: 204–212.

31. Rossini AL, Zacheo A, Mocini D, Totta P, Facchiano A, et al. (2008) HMGB1-stimulated human primary cardiac fibroblasts exert a paracrine action on human and murine cardiac stem cells. J Mol Cell Cardiol 44: 683–693.

32. Mitola S, Belleri M, Urbinati C, Coltrini D, Presta M, et al. (2006) Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. J Immunol. 176: 12-15.

33. Schlueter C, Weber H, Meyer B, Rogalla P, Bullerdiek J, et al. (2005) Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule. Am J Pathol. 166: 1259-1263.

34. Syed IS, Sanborn TA, Rosengart TK. (2004) Therapeutic angiogenesis: a biologic bypass. Cardiology. 101: 131-143.

35. Tang JM, Wang JN, Zhang L, Zheng F, Yang JY, et al. (2011) VEGF/SDF-1 promotes cardiac stem cell mobilization and myocardial repair in the infarcted heart. Cardiovasc Res 91: 402–411.

36. Takahashi M. (2008) High-mobility group box 1 protein (HMGB1) in ischaemic heart disease: beneficial or deleterious? Cardiovasc Res. 80: 5-6.

FIGURE LEGENDS

Fig. 1 Flow cytometric gating to identify endothelial progenitor cells (EPCs). (**A-D**) Cells in the granulocyte-differentiation antigen-1 (GR1)-negative and green fluorescent protein (GFP)-positive fraction were examined for the expression of CD34 and CD144. Cells positive for both of these antigens (double positive cells) were EPCs. (**E**) The ratio of EPC to GR1-negative cells. # $p < 0.05$ and ## $p < 0.01$ vs. pre-myocardial infarction (MI) in the same strain, ** $p < 0.01$ vs. wild-type (WT) mice post-MI ($n = 10$ for each groups)

Fig. 2 Plasma concentrations of HMGB1 4 days after MI (**A**) and HMGB1 levels in heart tissue (**B**). Plasma HMGB1 levels in the MI-operated mice were higher than those in the sham-operated mice in both WT and HMGB1-TG mice. Plasma HMGB-1 levels increased significantly in the HMGB1-TG mice compared to WT mice after MI. Cardiac HMGB1 levels in the WT mice did not significantly differ in the border zone and remote zone of MI. In the HMGB1-TG mice, levels of HMGB1 in the border zone

of MI were significantly lower than in the remoter zone of MI. ‡ $p < 0.05$, ‡‡ $p < 0.01$ vs. sham-operated mice, * $p < 0.05$, ** $p < 0.01$ vs. WT mice, † $p < 0.05$ vs. remote zone of MI in HMGB1-TG mice ($n = 10$ for each group)

Fig. 3 Infarct size of the WT and HMGB1-TG mice. (**A**) Representative histology of Masson–trichrome-stained heart cross-sections 4 weeks after coronary ligation. Scale bars show 1.0 mm. (**B**) Percentage of infarct size 4 weeks after coronary ligation was significantly smaller in the HMGB1-TG mice than in the WT mice. **(C)** Immunohistochemical staining with anti-PECAM1 antibody in the border zone of the MI. Scale bars show 50 μm. (**D**) The number of PECAM1-positive cells was significantly higher in the HMGB1-TG mice than in the WT mice. (**E**) Immunohistochemical staining with anti-αSMA antibody in the border zone of the MI. Scale bars show 50 μm. (**F**) The number of αSMA-positive cells was significantly higher in the HMGB1-TG mice than in the WT mice. * $p < 0.05$, ** $p < 0.01$ vs. WT mice ($n = 10$ for each group)

Fig. 4 Myocardial immunofluorescence staining of border zone 4 weeks after myocardial infarction. (**A**) green fluorescent protein (GFP), (**B**) platelet endothelial cell adhesion molecule-1 (PECAM1), (**C**) 4' 6-diamidino-2-phenylindole (DAPI) merged with GFP and PECAM1, (**D**) merged image with GFP and PECAM1, and (**E**) hematoxylin-eosin (HE) stain in WT mice. (**F**) GFP, (**G**) PECAM1, (**H**) DAPI merged with GFP and PECAM1, (**I**) merged image with GFP and PECAM1, and (**J**) HE stain in HMGB1-TG mice. Scale bars represent 50 μm. (**K**) The ratio of GFP and PECAM1 double-positive cells to all cardiac cells was significantly higher in the HMGB1-TG mice than in the WT mice. (n = 10 in each group) (**L**) The ratio of GFP and PECAM1 double-positive cells to PECAM1 positive cells was significantly higher in the HMGB1-TG mice than in the WT mice. * $p < 0.05$, ** $p < 0.01$ vs. WT mice (n = 10 in each group)

Fig. 5 Protein levels of VEGF (**A**) and angiopoietin (**B**) in heart tissue 1 week after MI. Cardiac levels of VEGF in the HMGB1-TG MI mice were significantly higher than those in the WT-MI mice and sham-operated mice, both in the border zone of infarction

and the remote zone. In contrast, cardiac angiopoietin levels were higher in border zone than in the remote zone in both the HMGB1-TG MI and WT MI mice, with no difference between HMGB1-TG-MI and WT-MI mice. ‡ $p < 0.05$, ‡‡ $p < 0.01$ vs. sham-operated mice, * $p < 0.05$ vs. WT mice and # $p < 0.05$ vs. remote zone ($n = 10$ in each group)

Fig.6 VEGF receptor 2 gene expression levels by reverse transcriptase-polymerase chain reaction. Expression levels were normalized to the GAPDH levels and expressed as fold increase over the level in the sham-operated WT mice. The expression levels of VEGF receptor 2 were higher in the MI-operated mice than in sham-operated mice in both WT and HMGB1 mice. Furthermore, the mRNA expression levels of VEGF receptor 2 in both border and remote zone in the HMGB1-TG mice were higher than those in the WT mice. ‡ $p < 0.05$, ‡‡ $p < 0.01$ vs. sham-operated mice, * $p < 0.05$, ** $p < 0.01$ vs. WT mice, ($n = 8$ in each group).